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Laboratory Guidebook
Notice of Change

Chapter **new**, revised, or archived: MLG 5C.00

Title: Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STECs) from Meat Products and Carcass and Environmental Sponges

Effective Date: 02/04/19

Description and purpose of change(s):

This method incorporates a rapid screen molecular assay for *stx* and *eae* detection and O group identification of the Top Seven Shiga Toxin-Producing *Escherichia coli* (STECs), merging the MLG 5 and MLG 5B procedures. Details for the screening assay are included in the equipment section. Rapid screen assay instructions and interpretations are included in the procedure.

A presumptive and confirmatory PCR screen procedure is included to characterize suspect STEC isolates found using mRBA and SBA.

Whole genome sequencing is included as an option to further characterize STEC isolates with inconclusive results during confirmation.

New appendices associated with the method include MLG 5C Appendix 1, MLG 5C Appendix 2, MLG 5C Appendix 3, MLG 5C Appendix 4, and MLG 5C Appendix 5.

Archived chapters include MLG 5, MLG 5 Appendix 2, MLG 5A, MLG 5B, MLG 5B Appendix 1, MLG 5B Appendix 2, MLG 5B Appendix 3, and MLG 5B Appendix 4.

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Title: Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges		
Revision: Original	Replaces: NA	Effective: 02/04/2019

Procedure Outline

5C.1 Introduction

The methods described in this guidebook are for use by the FSIS laboratories. FSIS does not specifically endorse any of the mentioned test products and acknowledges that equivalent products may be available for laboratory use. FSIS utilizes the following performance criteria when evaluating the suitability of an alternative laboratory method or product for a given analyte and sample matrix pair:

- Sensitivity of 90% or greater
- Specificity of 90% or greater
- Accuracy of 90% or greater
- Positive predictive value of 90% or greater
- Negative predictive value of 90% or greater

Performance criteria are relative to the reference cultural method for that analyte and sample matrix as outlined in the corresponding MLG chapter. Method validation is necessary to demonstrate the equivalence of alternative tests as detailed in the document titled "FSIS Guidance for Evaluating Test Kit Performance."

The following methods are used for the analysis of raw and ready-to-eat meat products, and laboratory environmental sponge samples for the seven major STEC serogroups (O157, O26,

O45, O103, O111, O121 and O145). The methods are based on enrichment in a selective broth medium, application of a rapid screening test, immunomagnetic separation (IMS) in paramagnetic columns, and plating on a highly selective medium, modified Rainbow Agar (mRBA). Colonies on mRBA are tested for the presence of O antigens using an agglutination test. Agglutination positive colonies are then streaked onto tryptic soy agar with 5% sheep blood (SBA) for confirmation using genetic assays and biochemical identification. Shiga Toxin-Producing *Escherichia coli* (STEC) refers to all seven serogroups in this method unless otherwise noted.

Unless otherwise stated all measurements cited in this method have a tolerance of ± 2%.

5C.2 Safety

STECs are categorized as a Biosafety Level 2 human pathogen with a low infectious dose (ingestion of 100 cells can cause disease). CDC guidelines for manipulating Biosafety Level 2 pathogens should be followed whenever live cultures of shiga toxin positive *E. coli* are used. The use of gloves and eye protection is recommended for all post enrichment viable culture work. Work surfaces should be disinfected prior to and immediately after use. A Class II laminar flow biosafety cabinet is recommended for activities in which infectious aerosols or splashes may be created. The Safety Data Sheets (SDS) must be obtained from the manufacturer for the media, chemicals, reagents and microorganisms used in the analysis. The personnel who will handle the materials should read all SDS.

5C.3 Quality Control Practices

5C.3.1 Method Controls

The control cultures should be started at the same time as and analyzed in the same manner as the samples. Confirm at least one isolate from the positive control sample. However, for safety considerations, toxin-attenuated or toxin-negative strains that have a typical appearance on mRBA may be used as controls on plating media for serological agglutination testing. In the absence of a positive test sample, control cultures may be terminated at the same point as the sample analyses.

A tagged *E. coli* O157:H7 reference strain that visibly fluoresces under ultraviolet (UV) light is used as a positive control along with an uninoculated media control that serves as a negative control for the enrichment process. A kit positive control that contains the DNA of all 7 STEC strains (*stx*+, *eae*+) and a kit negative control is used based on the requirements of the screening technology. If the screening technology used has no kit required controls that contain all 7 STEC strains (stx+, eae+) then prepare a DNA template positive PCR

control including the DNA of all 7 STEC strains (*stx*+, *eae*+). The top 7 *stx*+ and *eae*+ STEC control strains shall include *E. coli* O157:H7, *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *E. coli* O121, and *E. coli* O145. Such strains can be obtained through reference culture collection centers including but not limited to the American Type Culture Collection (ATCC), the STEC Center at The Michigan State University and the *E. coli* Reference Center at The Pennsylvania State University.

a. Sample Enrichment Controls

The tagged *E. coli* O157:H7 reference strain that visibly fluoresces under ultraviolet (UV) light will differentiate QC culture control strains from true contaminants. The control will be included with each sample batch as a positive control. The positive control culture will be inoculated into an appropriate matrix free of the target analyte at a low inoculum level, e.g., by preparing a test organism suspension in broth or saline, equivalent in turbidity to a 0.5 McFarland standard. Using a 1µl loop, inoculate the broth or streak the plates to be tested. Alternatively, commercially prepared bacterial pellets may be used.

An uninoculated media control will be included with each sample batch as a negative control.

b. STEC Controls

The *stx/eae* and serogroup-specific PCR controls include:

- Positive control - enrichment inoculated with the *E. coli* O157:H7 reference control strain.
- Negative control - uninoculated enrichment.
- Kit positive control or DNA template positive PCR control including the DNA of all 7 STEC strains (*stx*+, *eae*+).
- Kit negative control if required by the technology.

To prepare a DNA template positive PCR control, grow the top 7 STEC cultures on SBA and incubate at 35±2°C for 16-24 h. Pick typical colonies to create culture suspensions in molecular grade water corresponding to approximately 10^9 CFU/ ml (approximately 0.5 McFarland reading on the Dade Microscan or equivalent). In one tube, add 1.0 ml from each suspension to 3.0 ml of molecular grade water to create a 10.0 ml cocktail of all 7 strains. This will provide approximately a 10^8 CFU/ml cocktail using each strain. Aliquots of the suspension are then transferred to PCR tubes or microcentrifuge tubes and heated at 95-99°C

for 10 minutes on a thermocycler or heating block. The tubes shall be centrifuged at 10,000 x g for 3 minutes to pellet cellular debris. The supernatant may be used as the PCR positive control. DNA control template can be prepared as a batch, transferred to smaller volume tubes, and stored at ≤ -20°C for 1 year.

c. STEC IMS and Plating Controls

- Positive Control – use the enrichment inoculated with the *E. coli* O157:H7 reference control strain
- Negative Control - the uninoculated enrichment
- Streak an isolate from the serogroup(s) of interest (based on serogroup-specific PCR results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

5C.4 Equipment, Materials, Media, Reagents and Test Kits

5C.4.1 Equipment

a. Balance, sensitivity to at least ± 0.1 g
b. Blending/mixing equipment: Paddle blender, Sterile Osterizer-type blender with sterilized cutting assemblies, and blender jars or equivalent and adapters for use with Mason jars
c. Sterile plain, clear polypropylene bags with or without mesh (ca. 24" x 30 - 36"), or Whirl-Pak type bags (or equivalent)
d. Incubators, static 42 ± 1°C, 35 ± 2°C
e. For Bio-Rad iQ-Check™ rapid screen assay:
   i. Vortexer
   ii. Magnetic stir plate
   iii. Centrifuge
   iv. Bio-Rad real-time PCR System (CFX96)
   v. iQ-Check™ STEC VirX Kit (Cat. No. 3578139)
   vi. iQ-Check™ STEC SerO Kit (Cat. No. 3578140)
   vii. Hard-Shell 96-Well PCR Plates (Barcoded, HSP9955XTU), PCR strips or equivalent
   viii. Pipets and sterile filter tips for the 5uL, 20uL, 150uL to 1mL volumes
   ix. Optically Clear Heat Seal film (Cat. No. 1814030) and PX1 PCR Plate Sealer or PCR optical caps
   x. 1.5 or 2ml Sterile test tubes
   xi. Heating block (95-100°C)

xii. Specific for Extraction in Deep Wells:
- iQ-Check™ Deep Well Microplates (Cat. No. 3594900)
- Agitator-incubator for deep well plates (e.g. BioShake iQ Thermoshaker)
- Plastic sealing film
- Pre-pierced sealing film

xiii. Additional if using iQ-Check™ Prep System:
- 5mL Tube for PCR Mix (Cat. No. 3594901)
- 50uL Filter Tips (Cat. No. 3594902)
- 1000uL Filter Tips (Cat. No. 3594903)

f. Sterile inoculating loops, "hockey sticks" or spreaders, and needles
g. UV light (long-wave, e.g. VWR # 36553-124)
h. Filter unit, 0.2 µm, nylon, sterile
i. Infrared thermometer
j. Rotating tube agitator (e.g. LabQuake® Agitator) with clips to hold microcentrifuge tubes
k. Sterile disposable 12 x 75 mm polypropylene or polystyrene tubes (e.g. Fisher # 14-956-1B, Falcon® # 352063, or equivalent)
l. Microcentrifuge capable of speeds up to 16,000 x g and sterile microcentrifuge tubes (1.5- 2.0 ml)
m. Vortexer
n. Sterile 50 ml conical tubes (e.g. Falcon® # 2070) or sterile bottles
o. Sterile 40 µm Cell Strainer (Falcon® # 2340)
p. MACS® Large Cell Separation Columns (Miltenyi Biotec # 422-02)
q. OctoMACS® Separation Magnet (Miltenyi Biotec # 421-09)
r. Multistand to support OctoMACS® Separation Magnet (Miltenyi Biotec # 423-03)
s. Tray, autoclavable, approximately 130 mm x 83 mm (e.g. VWR # 62663-222) for use with the OctoMACS®
t. Biochemical test kit and system, GN cards (VITEK® 2 system, bioMerieux Vitek, Inc., 595 Anglum Drive, Hazelwood, MO 63042-2395)
u. Optical density reader
v. Romer Labs RapidChek® CONFIRM STEC Immunomagnetic Separation (IMS) Kit with anti-O26, anti-O103, anti-O111, anti-O145, anti-O45, and anti-O121 antibody-coated paramagnetic beads
w. RNase free, DNase free PCR Certified Water (Teknova # W3330)

5C.4.2 Media, Reagents and Cultures

a. Modified Tryptone Soy Broth (mTSB) or modified Tryptone Soy Broth with Novobiocin (mTSB+n)
b. Modified Rainbow® Agar (mRBA) [Rainbow® Agar O157 Biolog Inc., Hayward California, 94545] containing 5.0 mg/L novobiocin, 0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite
c. Tryptic soy agar with 5% sheep blood (SBA)
d. 1.0 N Hydrochloric Acid (HCl)
e. E Buffer, (See Media and Reagents Appendix 1, Buffered Peptone Water, Bovine Albumin Sigma # A7906-500G and Tween-20®)
f. Physiological saline solution (0.85% NaCl)
g. Disinfectant (Lysol® I. C., 2.0% or equivalent)
h. Dynal® # 710.04 anti-*E. coli* O157 antibody-coated paramagnetic beads (Life Technologies Corporation, Carlsbad, CA 92008)
i. Microbiologics UV-BioTAG [TM] EC 43 derived from FDA ESC1177 catalog# 01227UV-V or equivalent
j. *E. coli* O26, *E. coli* O45, *E. coli* O103, *E. coli* O111, *E. coli* O121, and *E. coli* O145 meeting the following genetic characteristics: *stx*+ and *eae*+. [American Type Culture Collection (ATCC), the STEC Center at The Michigan State University or the *E. coli* Reference Center at The Pennsylvania State University]

5C.4.3 Test Kits

a. Abraxis® non-O157 STEC Latex Agglutination Test (LAT) Kits or equivalent, specific for serogroups O26, O45, O103, O111, O121, O145
b. *E. coli* O157:H7 latex agglutination test kit (Abraxis® *E. coli* O157:H7 Latex Agglutination Test, Abraxis®, 54 Steamwhistle Drive, Warminster, PA 18974 [PN 541070]) or equivalent
c. BioRad® iQ-Check™ STEC VirX Kit (3578139)
d. BioRad® iQ-Check™ STEC SerO Kit (3578140)

5C.5 Enrichment Procedure

a. Sample Preparation and Primary Enrichment

Note: Disinfect the surface of intact sample package(s) prior to opening.

i. For beef trim/raw ground beef components, raw ground beef and raw beef/pork or poultry mixes, prepare in a sterile strainer bag a single sample in enrichment broth with a 1:4 dilution (one portion of product in three portions of medium), e.g., 325 ± 32.5 g sample with 975 ± 19.5 ml mTSB broth. Pummel, blend or hand massage until clumps are dispersed.

ii. For environmental and carcass sponges with 10 ml of buffer, add 50 ± 5 ml of mTSB broth. For carcass sponges with more buffer, use a 1:6 ratio of mTSB (for example, a sponge with 25 ml of buffer will use 125 ml of enrichment broth) to each sponge sample. Pummel, blend or hand massage until well mixed.

iii. For outbreak-related samples:
Raw Product: randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. When possible avoid the exterior surfaces of non-intact samples. Place each 25 ± 1 g sub-sample in a sterile strainer bag and add 225 ± 4.5 ml of mTSB for a 1:10 dilution. Pummel, blend or hand massage until clumps are dispersed.

Fermented Sausage or Cooked Meat Products: randomly collect thirteen 25 ± 1 g sub-samples (total of 325 ± 13 g) that are representative of the entire sample. When possible avoid the exterior surfaces of non-intact samples. Place each sub-sample in a sterile strainer bag and add 225 ± 4.5 ml (1:10 dilution) of mTSB+n. Pummel, blend or hand massage until clumps are dispersed.

iv. For Most Probable Numbers (MPN) determination, follow MPN instructions given in the specific program protocol or see MLG Appendix 2, Most Probable Number Procedure and Tables.

b. Incubate all bags (static) with their contents for 15 to 24 h at 42 ± 1°C for mTSB or 15 to 22 h at 42 ± 1°C for products that use mTSB+n. Include a positive and uninoculated medium control for each group of samples tested.

5C.6 Rapid Screening Procedure for *stx/eae* and O group identification

Follow the rapid screening technology user guide for preparing reagents, performing the test, and reading the results. The equipment must be operated and all records documented according to laboratory work instructions.

a. Following incubation, screen all enriched samples on the iQ-Check™ STEC VirX screening (*stx, eae*) PCR Assay. Follow the User's Guide for preparing reagents, performing the PCR, and interpreting results, if applicable.

b. Samples that test negative for *stx* and/or *eae* on the STEC screening PCR shall be reported as negative. Samples that test positive for the STEC screening PCR (*stx, eae*) will be further analyzed for O group identification using iQ-Check™ STEC® SerO test. NOTE: Store sample lysates at 2-8°C after loading onto instrument.

c. Samples that test positive for the STEC screening (*stx, eae*) but negative for the 7 serogroups shall be reported as negative. If an O-group is positive, the sample shall be reported as a potential positive. Proceed with the isolation procedure.

d. Samples with inconclusive results for the screening (*stx, eae*) may be tested again using STEC screening assays using either the same lysate or preparing new lysate tubes.

e. Lysates may be sealed and stored for additional testing at 2-8°C for up to 1 week or at ≤20°C for one year.

f. For samples that are *stx/eae* positive but have an inconclusive result on the O group assays, proceed to the isolation procedure and analyze for the inconclusive O groups. Alternatively, the laboratory may investigate. Based on the findings, the laboratory may:
   - repeat the rapid screen analysis from the lysate step or
   - prepare new rapid screen lysate tubes and repeat the analysis.

g. In analytical runs where the positive control results are not positive, all samples are affected and an investigation shall be performed. Based on the findings the laboratory may:
   - repeat the rapid screen analysis from the lysate step
   - prepare new rapid screen lysate tubes and repeat the analysis
   - analyze all of the samples culturally.

h. If reanalysis is unsuccessful, repeat sample preparation from the sample reserve or discard the sample. If circumstances (e.g. a power outage or equipment failure) do not allow testing using the rapid screen system, the laboratory shall, if possible, continue cultural analysis of all affected samples.

5C.7 Isolation Procedure

Samples that are potentially positive from PCR screening shall be plated onto mRBA following IMS. In the isolation procedure, IMS beads shall be used for the specific serogroup identified by the serogroup PCR reaction (e.g. anti-O157 will be used for samples with screen results positive for O157). Since the iQ-Check™ STEC SerO kit provides a combined *E. coli* O103 and O145 PCR positive screen result, anti-O145 IMS beads and anti-O103 IMS beads must be used for *E. coli* O103/O145 potential positives.

a. Remove mRBA plates from 2-8ºC storage, allowing 4 plates for each screen-positive culture and control, with the exception of 2 plates for the fluorescent *E. coli* O157:H7 control. Be sure that plates have no visible surface moisture at the time of use. NOTE: For non-O157 STECs, streak a control isolate from the serogroup(s) of interest (based on serogroup-specific screen results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

b. Remove a bottle of E Buffer from 2-8ºC storage. Decant E Buffer for each positive serogroup identified in the sample and each control into a sterile tube or bottle and allow it to warm to at least 18ºC.

c. For each positive control, and screen-positive culture to be analyzed, keep in order and label 50 ml conical centrifuge tubes.

d. For each positive control, and screen-positive culture, also label two sterile 1.5 ml microcentrifuge tubes and four 12 x 75 mm capped tubes. For each set of 12 x 75 mm tubes, label one tube and add 0.9 ml E Buffer to three of the four tubes.

e. Place a 40 µm Cell Strainer on a labeled 50-ml conical centrifuge tube. Pipet 5 ± 1 ml of each control and enrichment culture into the respective Cell Strainer and collect at least 1.0 ml of filtrate.

f. Transfer 50.0 µl (or volume recommended by the manufacturer) of appropriate immunomagnetic capture beads determined by the serogroup PCR screen results to a sterile, labeled microcentrifuge tube.

NOTE: For *E. coli* O157:H7, prepare the Dynal® #710.04 *E. coli* O157:H7 immunomagnetic bead suspension by following Table 1 below. Be sure to include the negative and positive control in the total number of cultures. Vortex the bead solution briefly (2-3 seconds), then add 50 µl to a labeled microcentrifuge tube, one

for the control and each screen-positive culture. Use bead suspension immediately or hold these tubes at 2-8ºC.

g. Transfer 1.0 ml of a filtrate to the corresponding microcentrifuge tube containing the immunomagnetic bead suspension and place in the clips of the LabQuake® tube agitator. Rotate the tubes for 10-15 min at 18-30°C.

h. Attach the OctoMACS® Magnet to the Multistand.

i. Position a tray on the base of the Multistand so that it will collect the filtrate passing through the columns. Add approximately 300 ml of 2% Lysol® I. C. or equivalent disinfectant to cover the bottom of the tray.

j. Label and place the appropriate number of Large Cell Separation columns on the OctoMACS® Magnet. Insert columns from the front, making sure the column tips do not touch any surfaces. Leave the plungers in the bags at this time to maintain sterility.

k. Transfer at least 0.5 ml E Buffer to the top of each column and let the buffer run through, then transfer each culture and control to its corresponding column.

l. After a culture or control has drained through, wash the column by applying 1.0 ml of E Buffer to each column and allow to drain. Repeat 3 more times for a total of 4 washes.

m. After the last wash has drained, remove the column from the OctoMACS® Magnet and insert the tip into an empty labeled 12 x 75 mm tube. Apply 1.0 ml of E Buffer to the column, and using the plunger supplied with the column, ***immediately*** flush out the beads into the tube. Use a smooth, steady motion to avoid splattering. Cap the tubes. Repeat this for each column. Repeat prep steps for the additional cultures.

n. Make a 1:10 dilution of each treated bead suspension by adding 0.1 ml of the bead suspension to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer. Make a 1:100 dilution by adding 0.1 ml of the 1:10 dilution to a 12 x 75 mm labeled tube containing 0.9 ml E Buffer.

o. Vortex briefly to maintain beads in suspension and plate 0.1 ml from each tube (1:10 dilution and 1:100 dilutions) onto a labeled mRBA plates. Use a hockey stick or spreader to spread plate the beads.

p. **Acid Treatment:** For each sample, transfer 450 µl of the undiluted bead suspension (MACS column eluant) to an empty labeled microcentrifuge tube. Add 25 µl of 1N hydrochloric acid (HCl) to this bead suspension and vortex briefly. This will bring the pH to 2.0-2.5 using E-buffer. Note: For the *E. coli* O157 serogroup, the fluorescent *E. coli* O157:H7 control sample is excluded as it does not grow following acid treatment.

q. Place the microcentrifuge tubes containing the acid treated suspension on a LabQuake® Agitator and rotate tubes for 1 hour at 18-30°C.

r. Dilute the suspension by adding 475 µl of E buffer.

s. Vortex briefly to maintain beads in suspension and plate 0.1 ml of the diluted suspension onto a labeled mRBA plate. Use a hockey stick or spreader to spread plate the beads.

t. Add 0.1 ml of the suspension to a labeled tube containing 0.9 ml E buffer and vortex briefly, representing a 1:10 dilution of the acid-treated cell suspension. Plate 0.1 ml of the diluted suspension onto a labeled mRBA plate.

u. As soon as there is no visible moisture on the agar surface, invert plates and incubate for 20-24 h at 35 ± 2°C.

v. Decontaminate the OctoMACS® Magnet by applying 2% Lysol® I. C. or equivalent disinfectant directly to the surface. After approximately ten minutes, rinse with deionized or tap water. Allow the unit to air-dry or use absorbent paper towels to dry the unit.

Table 1. Immunomagnetic Bead Suspension Volumes for *E. coli* O157

# of Cultures	*µl of Beads**	*µl of E-Buffer*	*# of Cultures*	*µl of Beads**	*µl of E-Buffer*
1	15	135	11	65	585
2	20	180	12	70	630
3	25	225	13	75	675
4	30	270	14	80	720
5	35	315	15	85	765
6	40	360	16	90	810
7	45	405	17	95	855
8	50	450	18	100	900
9	55	495	19	105	945
10	60	540	20	110	990

* Dynal® anti-*E. coli* O157:H7 antibody-coated paramagnetic beads (vortex briefly before use)
NOTE: Use a 1:10 dilution of stock beads for larger sample numbers

5C.8 Identification and Confirmation

5C.8.1 Identification of STECs

a. After incubation of mRBA, plates are to be examined for colonies that agglutinate with latex agglutination reagents specific for the serogroup of interest. Perform serological agglutination assays for STECs following manufacturer's instructions.

E. coli O157:H7 colonies typically have black or gray coloration on modified Rainbow® Agar. When *E. coli* O157:H7 colonies are surrounded by pink or magenta colonies, they may have a bluish hue. These colonies may also appear lighter, especially when plates are examined prior to 24 hours incubation. Coloration of non-O157 *E. coli* colonies may vary based on proximity to other competitor colonies or medium discoloration due to competitor colony growth, so test at least one colony from each identified colony morphology found on the mRBA plate. Colony colors from representative strains of STEC serogroups are listed in MLG 5C Appendix 2 "Morphologies of Representative Strains from Six non-O157 Shiga Toxin-Producing *Escherichia coli* (STEC) Grown on Modified Rainbow Agar."

Samples that have no growth on mRBA or colonies that are agglutination negative for the O group identified by the rapid screen can be reported as negative for STEC.

b. A presumptive rapid screen test is performed directly on agglutination positive colonies from the mRBA to verify presumptive positive colonies using the following procedure for the iQ-Check™ Assay:

- Transfer the remainder of an agglutination positive colony from the mRBA plate into 50 µl of Molecular Grade water and homogenize using a vortex (for up to 5 colonies).
- Add 5 µl of the suspension to 20 µl of PCR mix in a PCR strip or PCR plate. Continue with the manufacturer instructions for the iQ-Check™ STEC VirX and STEC SerO Assays.
- The sample is considered negative if the rapid screen is negative for the PCR targets.

- If an agglutination positive colony from mRBA is positive for *stx*, *eae* and O group, the sample is considered a presumptive positive for the identified STEC.
- From the previous suspension, streak SBA for isolation. Incubate inoculated SBA plates at 35 ± 2°C for 16-24 hours.

5C.8.2 Confirmation

a. After incubation, examine the SBA plates for both purity and evidence of contamination. Cross contamination with a positive fluorescent *E. coli* O157 control can be identified by using long wave UV light. Only the positive control culture should fluoresce. If the SBA plates appear pure and uncontaminated, perform the following confirmatory tests:

i. Biochemical confirmation
Inoculate VITEK® 2 GN cards (if using VITEK® 2 Compact).

ii. O antigen confirmation
To confirm the absence or presence of O antigens, use a serological agglutination kit targeting the O group of interest. Use growth from the SBA plate. For inconclusive results, genetic testing may be necessary.

iii. Shiga toxin gene confirmation
The presence of the *stx* and *eae* genes in any STEC culture isolate(s) is confirmed by the iQ-Check™ STEC VirX *stx/eae* Screening assay.

STECs are also confirmed by iQ-Check™ STEC® SerO Assay, targeting the O group of interest.

b. To perform a confirmatory test for the presence of *stx, eae* and O group, use the following procedure:

- After SBA incubation, perform the STEC serological agglutination test on colonies from the SBA plate.
- To confirm agglutination-positive colonies using iQ-Check™, transfer the remainder of an agglutination positive colony from the SBA plate into 50 µl of Molecular Grade water and homogenize using a vortex.

- Add 5 µl of the suspension to 20 µl of PCR mix in a PCR strip or PCR plate. Continue with the manufacturer instructions for the iQ-Check™ STEC VirX and STEC SerO Assays.
- Continue with the system protocol for the presence of *stx* and *eae* on the iQ-Check™ VirX Assay and the serogroup of interest on the iQ-Check™ SerO Assay.
- Additionally, perform biochemical identification (VITEK® 2) on agglutination positive colonies from the incubated SBA.

c. FSIS uses the following definitions for reporting a confirmed STEC positive:

- The sample is positive for non-O157 STEC if the isolate is agglutination positive for one or more of the six non-O157 STEC serogroups, positive for *stx* and *eae,* positive for one or more of the six non-O157 serogroup genes and biochemically identified as *E. coli*. If the isolate and any additional colony picks from mRBA are ultimately determined to be negative for either *stx, eae,* or top six serogroup genes, the sample is negative for non-O157 STEC.

- The sample is considered positive for *E. coli* O157 if the isolate is biochemically identified as an *Escherichia coli* isolate that is serologically and genetically determined to be "O157" and meets at least one of the following criteria:
  1) Positive for Shiga toxin (ST) production
  2) Positive for Shiga toxin gene(s) *(stx*)
  3) Genetically determined to be "H7"

Genetic testing for the detection of the H7 gene (e.g. PCR) is used for confirmation of biochemically identified *E. coli* isolates with a negative or inconclusive *stx* confirmation result. These isolates may be submitted for further Shiga toxin gene, H7 gene PCR testing, and/or whole genome sequencing (WGS). A *stx* negative *E. coli* O157 that is genetically determined to be "H7" is considered a confirmed positive. The sample is negative for *E. coli* O157 if the isolate and any additional colony picks from mRBA are ultimately determined to be negative for *stx* and the H7 gene.

d. If an isolate or any additional presumptive positive colony picks from mRBA that are inconclusive (e.g. biochemically negative but O group positive), then the isolates are submitted for WGS testing.

Table 2. Characteristics of typical vs atypical *E. coli* O157:H7 strains

Test	**Typical *E. coli* O157:H7/NM Strain**	**Atypical *E. coli* O157:H7/NM Strain**
Screening Assay Result	Positive	Negative
Plating Medium (mRBA)	Blue-grey	Pink or white
Latex Agglutination	Positive (O157)	Positive or Negative (O157)
Rapid Screen Assay Confirmation	Positive - O157 Positive – eae Positive/Negative – stx Positive – fliCH7 (real-time PCR) or WGS* *If applicable (stx negative)	Positive/Negative - O157 Negative – *eae* Negative – *stx* Negative – fliCH7 (real-time PCR) or WGS
Biochemical Identification	*Escherichia coli* (possible O157 due to lack of sorbitol fermentation – Sorbitol Negative)	*Escherichia coli* (ferments sorbitol – Sorbitol Positive)
Possible Serotype	*E. coli* O157:H7	*E. coli* O157:H12, *E. coli* O157:H28, non-O157 *E. coli*

5C.9 Storage of Cultures

Store "working" *E. coli* stock cultures on nutrient agar slants or BHI agar slants. Transfer stocks monthly onto duplicate nutrient or BHI agar slants, incubate overnight at 35 ± 2°C, and then store them at 2-8°C. Use one of the slants as the working culture. Use the other slant for sub-culturing to reduce the chances of contamination. After five transfers, the culture must be re-confirmed biochemically or a new culture initiated. For long term storage, freeze cultures at ≤ -70C or less using cryo-beads i.e. Cryostor™ or lyophilize.

5C.10 Selected References

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iQ-Check™ STEC SerO User Guide, Test for the real-time PCR detection of 7 major serogroups in Shiga Toxin Producing *E. coli*